Video Article

Intracerebroventricular and Intravascular Injection of Viral Particles and Fluorescent Microbeads into the Neonatal Brain

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Abstract

In the study on the pathogenesis of viral encephalitis, the infection method is critical. The first of the two main infectious routes to the brain is the hematogenous route, which involves infection of the endothelial cells and pericytes of the brain. The second is the intracerebroventricular (ICV) route. Once within the central nervous system (CNS), viruses may spread to the subarachnoid space, meninges, and choroid plexus via the cerebrospinal fluid. In experimental models, the earliest stages of CNS viral distribution are not well characterized, and it is unclear whether only certain cells are initially infected. Here, we have analyzed the distribution of cytomegalovirus (CMV) particles during the acute phase of infection, termed primary viremia, following ICV or intravascular (IV) injection into the neonatal mouse brain. In the ICV injection model, 5 µl of murine CMV (MCMV) or fluorescent microbeads were injected into the lateral ventricle at the midpoint between the ear and eye using a 10-µl syringe with a 27 G needle. In the IV injection model, a 1-ml syringe with a 35 G needle was used. A transilluminator was used to visualize the superficial temporal (facial) vein of the neonatal mouse. We infused 50 µl of MCMV or fluorescent microbeads into the superficial temporal vein. Brains were harvested at different time points post-injection. MCMV genomes were detected using the *in situ* hybridization method. Fluorescent microbeads or green fluorescent protein expressing recombinant MCMV particles were observed by fluorescent microscopy. These techniques can be applied to many other pathogens to investigate the pathogenesis of encephalitis.

Video Link

The video component of this article can be found at http://www.jove.com/video/54164/

Introduction

When studying viral encephalitis, the initial distribution of viral particles is very important to understand disease pathogenesis and to identify viral targets in the brain. Most viruses range in size from 20 to 300 nm, although the Pandoravirus is more than 700 nm in size[1]. The distribution of the viral particles in the acute phase of infection may depend on the size of the particles, the distribution of cellular receptors, or the affinity of the cellular receptors for viruses. In animal models, intracerebroventricular (ICV), intraperitoneal, direct placental, and intravenous (IV) infections have been used to study the pathogenesis of viral encephalitis. ICV inoculation with virus is often used to establish central nervous system (CNS) infections in mice. Studies using this technique report widespread infection, particularly of cells in the periventricular zones and in regions of the brain in direct contact with the cerebrospinal fluid (CSF), similar to the effects of viral ventriculoencephalitis. The small size of adeno-associated virus (AAV) particles (20 - 25 nm in diameter) facilitates their dissemination throughout the brain in ICV infections[2-4]. Intraperitoneal[5], direct placental[6], and IV injections[7] represent hematogenic systemic administration. The penetration of viral particles through the blood-brain barrier (BBB) allows them to reach the parenchyma of the neonatal brain, representing diffuse microglial nodules[8,9].

Cytomegalovirus (CMV) is a common virus that belongs to the herpes virus family. In the United States, 50% - 80% of the people have had CMV infection by age 40. CMV infections are rarely harmful but can cause diseases in immunocompromised patients and fetuses. Of all deliveries, 0.2% - 2% are born with CMV[10], resulting in severe symptoms such as microcephaly, periventricular calcification, cerebellar hypoplasia, microphthalmia, and optic nerve atrophy[11,12]. Furthermore, mental retardation, sensorineural hearing loss, visual defects, seizure, and epilepsy occur in about 10% of non-fatally CMV-infected infants[13,14]. CNS dysfunction is the most common characteristic symptom of CMV congenital anomaly. More children are permanently disabled each year by congenital CMV than by Down syndrome, fetal alcohol syndrome, or spina bifida[15]. There are no vaccinations against CMV available at the present, calling for a need of a safe and effective vaccine. Studying the interaction of CMV particles with their receptors in the earliest phase of infection is important to understand the effect of vaccination.

Ventriculoencephalitis and diffuse microglial nodules are the two main pathological characteristics of CMV encephalitis[16]. It has been uncertain how the CMV particles (150 - 300 nm) spread through the brain in the acute phase of infection and how the distribution of cellular receptors and their affinity for viruses contribute to the viral spread. Kawasaki *et al.* have evaluated ICV and IV infections from the perspective of the distribution of particles and their receptors (β1 integrin) in the earliest phase of infection. We have found that the dissemination of CMV particles and the expression of β1 integrin are well correlated in the earliest phase of infection in both ICV and IV infections[8]. ICV infection is a model of

ventriculoencephalitis and IV infection is a model of diffuse microglial nodules. Studying the dynamics of viral or fluorescent particles would give useful information on the effect of particle size, viral interactions with cellular receptors, and the mechanism of BBB penetration in the brain. The following protocol could be used to investigate any viral infection and viral vector in the CNS.

Protocol

All the experimental protocols were approved by the Animal Care Committee of Hamamatsu University of School of Medicine.

1. Preparation of MCMV (Smith strain) and Recombinant M32-enhanced Green Fluorescent Protein (EGFP)-MCMV

1. Generate recombinant M32-EGFP-MCMV according to the method as follows (1.2 - 1.9) and as previously described[8].
2. Use recombinant viruses derived from the Smith strain of wild-type MCMV (accession number: U68299). Insert EGFP (4,361 base pairs; bp) between 37,089 and 41,450 bp (*M32* - *M31* locus) in the MCMV genome by homologous recombination.
3. Amplify by polymerase chain reaction the MCMV *M32* (41,450 - 39,286 bp, left flanking sequence and *M31* (37,089 - 39,246 bp, right flanking sequence gene loci using the following primers: *M32*, 5′-CTACTAGCTAGCCTTCCGCGAGTCGCTGTATT-3′ (forward primer), 5′-CTACTAGCTAGCCTTCCGCGAGTCGCTGTATT-3′ (reverse primer); *M31*, 5′-CTAAATTAACTTAAGTCGTCCTCTTCGTCTCACAA-3′ (forward primer), 5′-AACTAGTCTAGATCGCTCCTGGTTGGTTTTTA-3′ (reverse primer).
4. Insert the *M32* locus into the EGFP-expressing vector using NheI and BamHI restriction sites. Insert the *M31* locus into the *M32-EGFP*-recombinant plasmid using AflII and XbaI sites. Cleave the *M32-EGFP-M31* sequence with NheI and AflII from the *M32-EGFP-M31* recombination plasmid and dissolve in H_2O.
5. Transfect the cleaved construct into the nuclei of MCMV (Smith strain)-infected NIH3T3 cells 24 hr post-infection (hpi) using an electroporation system to induce homologous recombination.
6. At 3 days post-infection, co-culture the cells with uninfected mouse embryonic fibroblasts (MEFs) at a ratio of 1/1,000 in six-well plates and screen for EGFP-expressing foci of infected cells.
7. Harvest the virus-containing supernatants from the wells containing green fluorescent foci and dilute tenfold. For purification, choose wells that display single green fluorescent foci.
8. When all foci resulting from the limiting-dilution infection display EGFP expression, the virus preparation is pure, indicating that no wild-type virus remains. Quantify the virus by the plaque-assay method as previously described[17].
9. Treat MCMV in certified biosafety cabinets at biosafety level 2 wearing gloves and mask.
10. Passage MCMV (Smith strain) and recombinant MCMV in MEFs prepared from 12-day-old ICR mouse embryos as previously described[17].
11. Remove cells from the supernatants of infected MEF cultures by centrifugation at 3,000 × g for 20 min at 16 °C.
12. Ultracentrifuge the supernatants for 40 min at 70,000 × g. Resuspend the pellets containing virions in 1 ml of Tris-buffered saline and transfer onto a preformed linear sorbitol gradient (25% - 70%). Ultracentrifuge again at 70,000 x *g* for 60 min[18].
13. Harvest the virion-containing band with a syringe. Pellet the harvested virions by an additional ultracentrifugation step at 70,000 × g for 40 min.
14. Resuspend the pellet in 1 ml of phosphate-buffered saline (PBS) and store at -80 °C until the infection experiments.
15. Quantify the virus titer by the plaque-assay method as previously described[19].
16. Visualize the EGFP expression of the recombinant MCMV particles (excitation at 489 nm, emission at 508 nm) by fluorescent microscopy (**Figure 1A**) and the particle structure by transmission electron microscopy (TEM)[8] (**Figure 1B**).

2. Preparation of Nile Red Fluorescent Microbeads

1. Purchase carboxyl fluorescent Nile red microbeads and place 500 µl of microbeads into tubes according to diameter (0.04 - 0.06 µm, approximately 3.63×10^{12} particles; 0.1 - 0.3 µm, approximately 5.75×10^{10} particles; and 1.7 - 2.2 µm, approximately 6.85×10^{7} particles).
2. Treat beads with 500 µl of 0.1 M NaOH for approximately 1 day to remove any endotoxins and resuspend the beads in sterile water at RT.
3. Adsorb the beads O/N with 10% mouse serum obtained from C57BL/6 mice at RT prior to use.
4. To separate aggregates, vortex the beads and sonicate thoroughly before use.

3. ICV Injection of MCMV and Fluorescent Microbeads into Neonatal Mice

1. Maintain normal pregnant ICR mice in a temperature-controlled facility under a 12 hr light/dark cycle. The neonates are designated as P 0.5 on the day of birth.
2. Sterilize a 10-µl syringe and a 27 G needle with 70% alcohol.
3. Load the injection solution (5 µl) containing MCMV or fluorescent microbeads into the needle by carefully pulling the plunger of the syringe.
4. Restrain neonatal mouse (P 0.5) by putting the mouse on ice for 3 - 4 min. Once the animal is under anesthesia, use the toe-pinch response method to determine the depth of anesthesia.
5. Mark the injection site with a non-toxic laboratory pen at a location approximately 0.7 - 1.0 mm lateral to the sagittal suture and 0.7 - 1.0 mm caudal from the neonatal bregma (**Figure 2A**).
6. Insert the needle 2 mm deep, perpendicular to the skull surface at the marked injection site. For reference, mark 2 mm from the tip of needle with a non-toxic maker.
7. Slowly inject 5 µl of MCMV (approximately 5×10^{5} PFU) into the lateral ventricle without opening the scalp (**Figure 2B**).
8. In another group of mice, inject a 5 µl solution containing fluorescent microbeads (0.1 - 0.3 µm, approximately 5.75×10^{8} particles) by the same method.
9. Slowly remove the needle 10 - 20 sec after discontinuing the plunger movement to prevent backflow.
10. To recover, keep the mice for 5 - 10 min in a warm container until movement and general responsiveness are restored.

11. Harvest the brains as described in section 5 at a range of time points (3, 12, 24, 48, and 72 hr) post-injection.

4. IV Injection of MCMV or Fluorescent Microbeads into Neonatal Mice

1. Restrain neonatal mouse (P 0.5) by putting the mouse on ice for 3 - 4 min.
2. Use a 1-ml syringe with a 35 G needle to perform the intravenous injection of MCMV in P 0.5 neonates.
3. Use a transilluminator (vein finder) to visualize the superficial temporal (facial) vein. Before injection, secure the neonate to the transilluminator using surgical tape (**Figure 2C**).
4. While wearing magnifying glasses (1.5X), slowly infuse 50 µl of MCMV (approximately 5.45×10^9 particles) or fluorescent microbeads (0.04 - 0.06 µm, approximately 3.63×10^{11} particles; 0.1 - 0.3 µm, approximately 5.75×10^9 particles; and 1.7 - 2.2 µm, approximately 6.85×10^6 particles) into the superficial temporal vein (**Figure 2D**).
5. After removing the needle, use gauze containing 70% alcohol to apply pressure to the injection site until the bleeding ceases.
6. Give the neonate approximately 5 min in a warm container to recover before returning it to the cage.
7. Harvest the brains as described in section 5 at a range of time points (3, 12, 24, and 72 hr) post-injection.

5. Brain Tissue Sample Preparation for Paraffin Sections

1. Place the injected neonates in a small plastic dish on crushed ice.
2. Once the animal is under anesthesia, use the toe-pinch response method to determine the depth of anesthesia.
3. Make one central or two end horizontal end cuts through the rib cage to open up the thoracic cavity.
4. Make a cut in the atrium with sharp scissors. Infuse 4% paraformaldehyde (PFA) solution into the atrium. Stop perfusion when spontaneous movement (PFA dance) and lightened-color of the liver are observed. Do not exsanguinate.
5. Dissect the neonate (as previously described[20]) by first removing the head using a pair of scissors.
6. Make a midline incision along the integument from the neck to the nose to expose the skull.
7. Place the sharp end of a pair of iris scissors into the foramen magnum on one side, carefully sliding along the inner surface of the skull.
8. Make a cut extending to the distal edge of the posterior skull surface, and make an identical cut on the contralateral side. Clear away the skull around the cerebellum.
9. Carefully peel back the skull on one side to prevent damage to the brain. Repeat this procedure on the other side of the brain.
10. Using a spatula, sever the olfactory bulbs and nervous connections along the ventral surface of the brain.
11. Gently separate the brain from the head, trimming any dura that still connect the brain to the skull using scissors, and remove the brain.
12. Place the brain in a vial of fixative containing 4% PFA at least 10 times the volume of the brain for 24 hr at 4°C.
13. Dehydrate the tissue through a series of graded ethanol baths to displace the water, and then infiltrate with paraffin wax, forming a block. Cut the paraffin block with a microtome into slices 4 µm thick[21].
14. Deparaffinize and rehydrate the slides of the infected brains[22]. Proceed to *in situ* hybridization for paraffin embedded sections.

6. Brain Tissue Sample Preparation for Frozen Sections

1. Remove the brain following the steps outlined in 5.1 - 5.11.
2. To observe the fluorescent microbeads and M32-EGFP-MCMV, place the resected brain on flat bottom cryomolds. Add embedding medium to completely cover the brain sample. Snap freeze the cryomolds in precooled *n*-hexane (-80 ºC), using forceps to hold the edge of the cryomolds prior to attaching it to the chuck.
3. For sectioning, attach the frozen tissue block on the precooled cryostat chuck. Transfer the frozen tissue with the chuck into a cryostat chamber and lower the temperature to between -10 and -20 °C, and cut the slices approximately 8 - 10 µm thick[23].
4. Fix the sections with cytofixative (mixture of isopropyl alcohol and polyethylene glycol) by spraying. Air dry the sections for 30 min at RT immediately after spraying, and store them at - 80 ºC until further use.
5. Equilibrate the sections back to RT and wash three times with PBS.
6. Stain the sections with fluorescein isothiocyanate (FITC)-conjugated *Griffonia simplicifolia* isolectin B4 at a concentration of 1:100 for 10 min in PBS at RT (**Figure 5**) or with PE-conjugated CD31 antibody at a concentration of 1:100 for 30 min in PBS at RT (**Figure 6**).
7. Wash the sections with PBS three times.
8. After the washing, stain the sections with 4′,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. Mount the sections in anti-fade reagent and image DAPI (excitation at 345 nm, emission at 455 nm), EGFP (excitation at 489 nm, emission at 508 nm), and Nile red (excitation at 553 nm, emission at 637 nm) with a fluorescent microscope (**Figures 3, 5, 6**).

7. Fluorescent *in situ* Hybridization (FISH) for Paraffin Embedded Sections

1. Prepare the FISH probe labeled directly with fluorophore for DNA *in situ* hybridization by nick translation using a bacterial artificial chromosome containing the whole MCMV DNA genome (pSM3fr)[19]. The concentration of the FISH probe is 0.1 µg/µl.
2. Deparaffinize and rehydrate the slides of the infected brains[22].
3. Treat the tissue sections with RNase (100 µg/ml in PBS) to detect viral DNA.
4. Perform antigen retrieval with a 0.05% NP40, 0.01 M citrate buffer (pH 6.0) at 95 - 98°C for 20 min. Cool the slides down to RT for 20 min.
5. In a glass Coplin staining jar, wash the slides in pure water three times for 2 min each time. Perform an additional antigen retrieval step with a 0.06% pepsin, 0.01 N HCl solution for 5 min at 37 °C.
6. Rinse slides three times in pure water for 2 min each time in a glass Coplin staining jar.
7. Dehydrate the tissue sections again by transferring the slides from 70% ethanol, to 85% ethanol, and then to 100% ethanol.
8. For preparing 10 ml of the hybridization buffer, mix 1.25 ml *in situ* hybridization salts (3 M NaCl, 100 mM Tris-HCl pH 8.0, 100 mM sodium phosphate pH 6.8, 50 mM EDTA) with 5 ml deionized formamide, 2.5 ml 50% Dextran sulfate, 250 µl 50x Denhardt's solution, 125 µl 100 mg/ml tRNA, and 875 µl H_2O.

9. Dilute the DNA probe directly labeled with fluorophores that recognize the whole MCMV genome randomly in the hybridization buffer. The final volume should be 10 µl (7 µl hybridization buffer, 1 µl probe (0.1 µg/µl), and 2 µl distilled water).
10. Add the probe mix (10 µl) to each slide and cover with a coverslip (15 × 15 mm). Seal the coverslip with rubber cement. Denature the probe mix for 5 min at 85 °C, and complete the hybridization step O/N at 42 °C.
11. Wash the slides with 0.3% NP40, 0.4x SSC at 73°C for 2 min; with 0.1% NP40, 0.4x SSC at 73 °C for 1 min; and with 2x SSC twice.
12. Counterstain the nuclei with DAPI (10 ng/ml) and cover the slide with a coverslip.
13. Mount the sections in anti-fade reagent and image DAPI (excitation at 345 nm, emission at 455 nm) and EGFP (excitation at 489 nm, emission at 508 nm) with a fluorescent microscope (**Figure 4**).

Representative Results

In studies on the pathogenesis of viral encephalitis, the infection method is important. The hematogenous route represents an acute infection of the endothelial cells and pericytes of the brain, while the ICV route represents an acute infection spreading via the CSF through the subarachnoid space, reaching to the meninges and choroid plexus. To analyze the first distribution of particles in acute encephalitis, *in situ* hybridization detecting the MCMV genomes and direct observation of M32-EGFP-MCMV particles or fluorescent microbeads were used.

Generation of Recombinant MCMV (M32-EGFP-MCMV)

The protocol illustrates how recombinant M32-GFP-MCMV was generated. EGFP was inserted between 37,089 and 41,450 bp (*M32-M31* locus) in the MCMV genome by homologous recombination. An EGFP protein was fused with a viral protein (M32) and EGFP was expressed within the viral particles. After homologous recombination, a virus preparation was considered pure when all foci resulting from the limiting-dilution infection displayed EGFP expression, indicating that no wild-type virus remained. It may take multiple dilution procedures to purify the recombinant virus. **Figure 1A** shows EGFP signals of purified recombinant MCMV (M32-EGFP-MCMV) and **Figure 1B** shows a TEM image of M32-EGFP-MCMV particles containing the virus genome in the viral core.

Intracerebroventricular Injection and Intravascular Injection

Figure 2 shows the injection sites of neonatal mouse (P 0.5) where virus or microbeads were injected through the ICV route or the IV route. In ICV, the injection site is at the midpoint between the ear and eye, at a location approximately 0.7 - 1.0 mm lateral to the sagittal suture and 0.7 − 1.0 mm caudal from the neonatal bregma (**Figure 2A**). The needle should be injected into the lateral ventricle 2 mm deep, perpendicular to the skull surface (**Figure 2B**). **Figure 2C** and **2D** show that MCMV or microbeads are injected into the temporal facial vein of neonatal mice. A transilluminator and magnifying glasses are helpful tools to clearly visualize small veins.

The Distribution of Viral Particles/Genomes and Fluorescent Microbeads

The following figures show the distribution of viral particles/genomes and fluorescent microbeads in the acute phase. **Figure 3** shows the microbead signals of frozen sections of the brain. **Figure 3A and 3B** show the distribution of the 0.1 - 0.3 µm fluorescent Nile red microbeads in the marginal area (MA) (**Figure 3A**) and the choroid plexus and subventricular zone (SVZ) (**Figure 3B**) at 2 hr post ICV injection. **Figure 3C and 3D** show the distribution of the 0.1 - 0.3 µm fluorescent Nile red microbeads in the MA and vascular area (**Figure 3C**) and the choroid plexus and SVZ (**Figure 3D**) at 2 hr post IV injection. It is likely that, following ICV and IV injection, particles did not immediately spread uniformly throughout the parenchyma. Rather, they stayed in the SVZ, MA, and vascular area in the acute infection phase. The size of the particles is the primary factor affecting their distribution in the brain in the acute phase following ICV and IV injection. **Figure 4** shows the FISH of the MCMV genome on the paraffin embedded sections of the brain. The MCMV genome (green spots) was detected in the SVZ at 3 hr post ICV injection (**Figure 4B**). The MCMV genomes (green spots) were detected in the MA and vascular area at 3 hr post IV injection (**Figure 4C**). The distribution of the MCMV(**Figure 4**) and microbeads (**Figure 3**) were quite similar. **Figure 5** shows the size-dependent distribution patterns of microbeads in the frozen sections of the brain following IV injection. Microbeads with diameters 1.7 - 2.2 µm (**Figure 5B**), 0.1 - 0.3 µm (**Figure 5D**), and 0.04 - 0.06 µm (**Figure 5E**) are shown respectively. Smaller microbeads had a tendency to be extravasated out of the vascular area in the parenchyma. **Figure 6** shows the distribution of M32-EGFP-MCMV particles in the frozen sections of the brain. GFP dot signals (M32-EGFP-MCMV particles) were mainly observed in the MA (**Figure 6A**) and choroid plexus and SVZ (**Figure 6B**) at 2 hr post ICV injection. The MCMV particles were found inside and outside of the vascular area of the parenchyma (**Figure 6C**) and choroid plexus and SVZ (**Figure 6D**). For M32-EGFP-MCMV particles, the rate of extravagation out of the vascular area was higher in the meninges and choroid plexus than in the parenchyma of the brain, indicating that the vessels of the MA and choroid plexus are more permeable than those of the parenchyma.

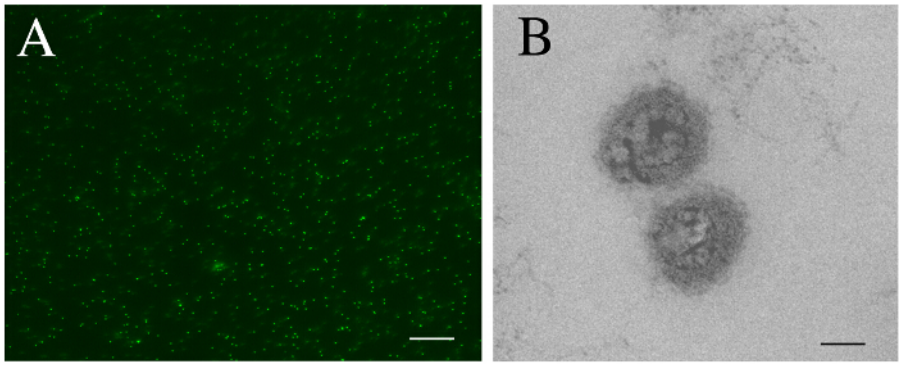

Figure 1: Generation of Recombinant M32-EGFP-MCMV. (A) Ultracentrifuged M32-EGFP-MCMV particles are seen as green spots by fluorescent microscopy. Scale bar: 2.4 µm. **(B)** Transmission electron microscopy reveals that M32-EGFP-MCMV particles possess a typical virus structure. Scale bar: 77 nm. Please click here to view a larger version of this figure.

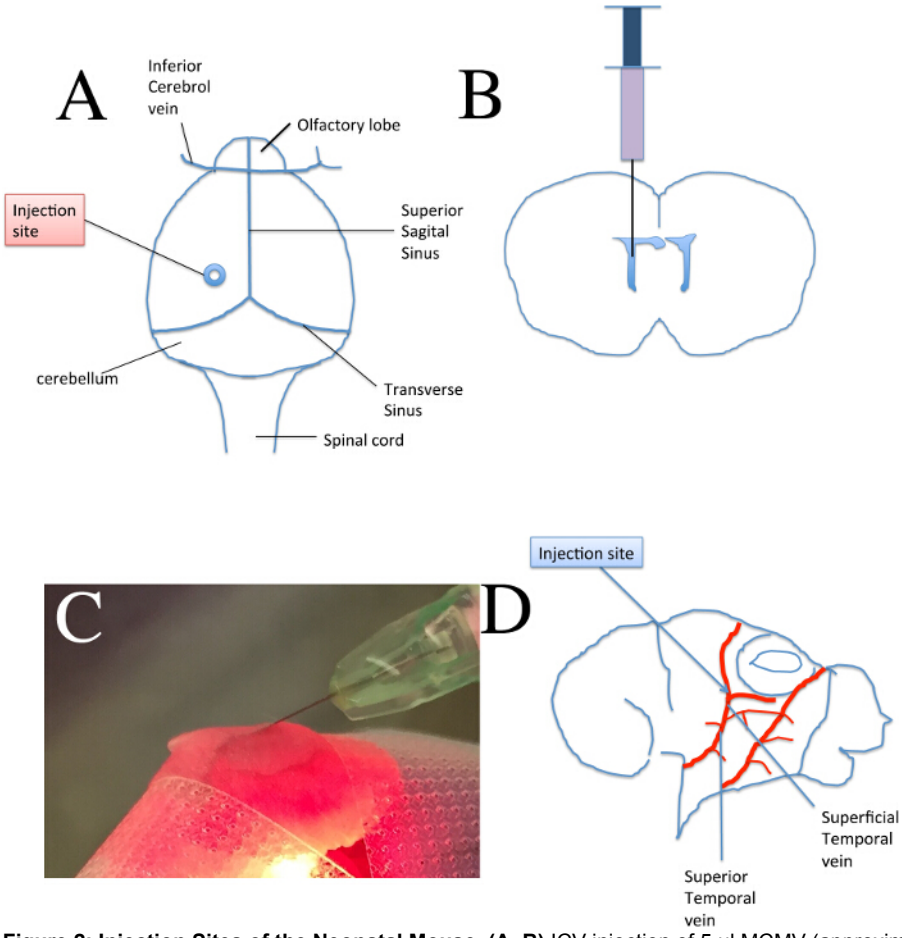

Figure 2: Injection Sites of the Neonatal Mouse. (A, B) ICV injection of 5 µl MCMV (approximately 5×10^5 PFU) or of 5 µl microbeads (approximately 5.75×10^9 particles). **(A)** The injection site is at the midpoint between the ear and eye (at a location approximately 0.7 - 1.0 mm lateral to the sagittal suture and 0.7 - 1.0 mm caudal from the neonatal bregma). **(B)** A 27 G needle with a 10-µl syringe is used for the injection into the lateral ventricle 2 mm deep, perpendicular to the skull surface. **(C, D)** MCMV or microbeads are injected into the temporal facial vein of neonatal mice. **(C)** A transilluminator (vein finder) is used to visualize the superficial temporal (facial) vein. (**D)** A 1-ml syringe with a 35 G needle is used to perform the IV injection of MCMV. Please click here to view a larger version of this figure.

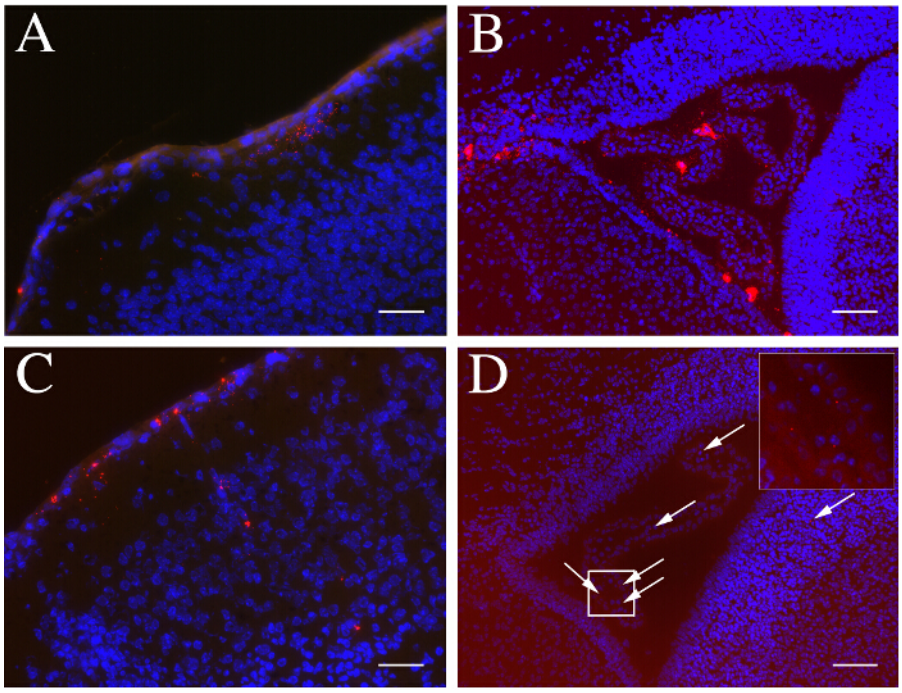

Figure 3: Visualization of Fluorescent Nile Red Microbeads in the Brain. (A, B) 0.1 - 0.3 µm fluorescent Nile red microbeads signals (red) are observed in the MA **(A)** and the choroid plexus and SVZ (**B)** at 2 hr post ICV injection. **(C, D)** 0.1 - 0.3 µm fluorescent Nile red microbeads introduced into the neonatal mouse brain by IV injection at 2 hr post injection. Nile red microbeads are observed in the MA and vascular area **(C)** and the choroid plexus and SVZ **(D)**. The white square is magnified in the enlarged insert in the top right of **(D)**. **(A, C)** Scale bar: 50 µm. **(B, D)** Scale bar: 150 µm. Arrows indicate Nile red microbeads. Please click here to view a larger version of this figure.

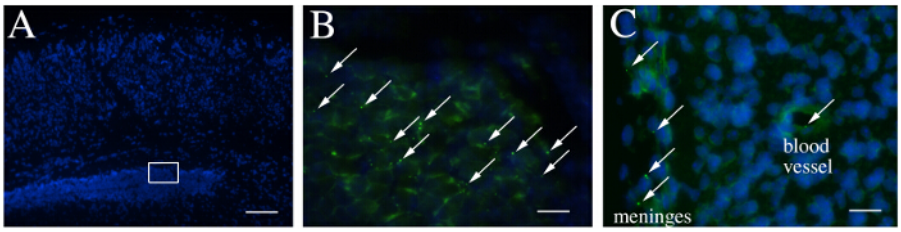

Figure 4: MCMV Genome Distribution in the Mouse Brain Following ICV Injection and IV Injection. (A) The lower magnification view. The white square indicates where the magnified image **(B)** was captured. **(B)** The MCMV genome (green spots) is first detected in the SVZ at 3 hr post ICV injection. DAPI (4′,6-diamidino-2-phenylindole) is used for nuclear acid (nuclear) staining. Scale bar: 30 µm. **(C)** The MCMV genome (green spots) is detected in the vascular area and meninges at 12 hr post IV injection. DAPI is used for nuclear acid staining. Scale bar: 30 µm. Arrows indicate representative MCMV genome (green spots). Please click here to view a larger version of this figure.

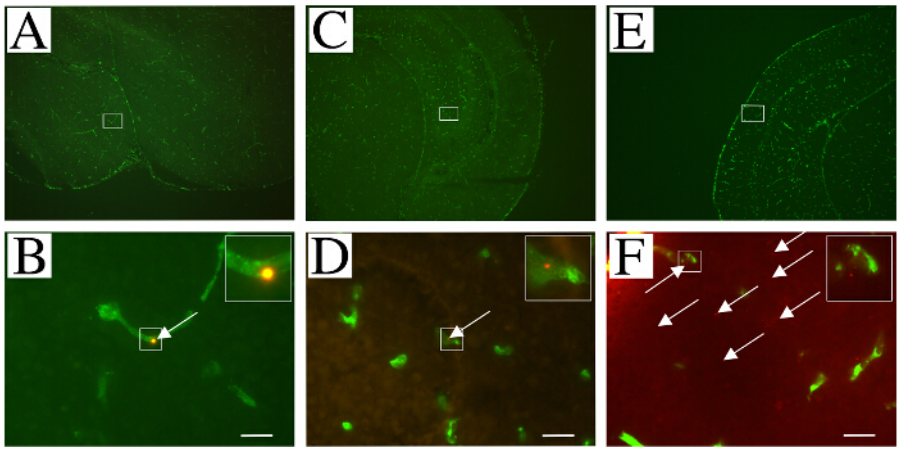

Figure 5: The Relationship between the Diameter of Microbeads and the Distribution of the Microbeads. Fluorescent Nile red microbeads (50 µl) are introduced into neonatal mouse brains by IV injection. In frozen sections prepared 2 hr post injection, Nile red signals (red, indicated by arrows) are observed in and around the vascular area of the parenchyma. The vascular area is stained with fluorescent lectin (green). **(A, B)** 1.7 - 2.2 µm microbeads. **(A)** The lower magnification view. The white square indicates where the magnified image **(B)** was captured. **(B)** The white square is magnified as the enlarged insert in the top right of **(B)**. **(C, D)** 0.1 - 0.3 µm microbeads. **(C)** The lower magnification view. The white square indicates where the magnified image **(D)** was captured. **(D)** The white square is magnified as the enlarged insert in the top right of **(D)**. **(E, F)** 0.04 - 0.06 µm microbeads. **(E)** The lower magnification view. The white square indicates where the magnified image **(F)** was captured. **(F)** The white square is magnified as the enlarged insert in the top right of **(F)**. Scale bar: 8 µm. Arrows indicate representative microbeads (red spots). Please click here to view a larger version of this figure.

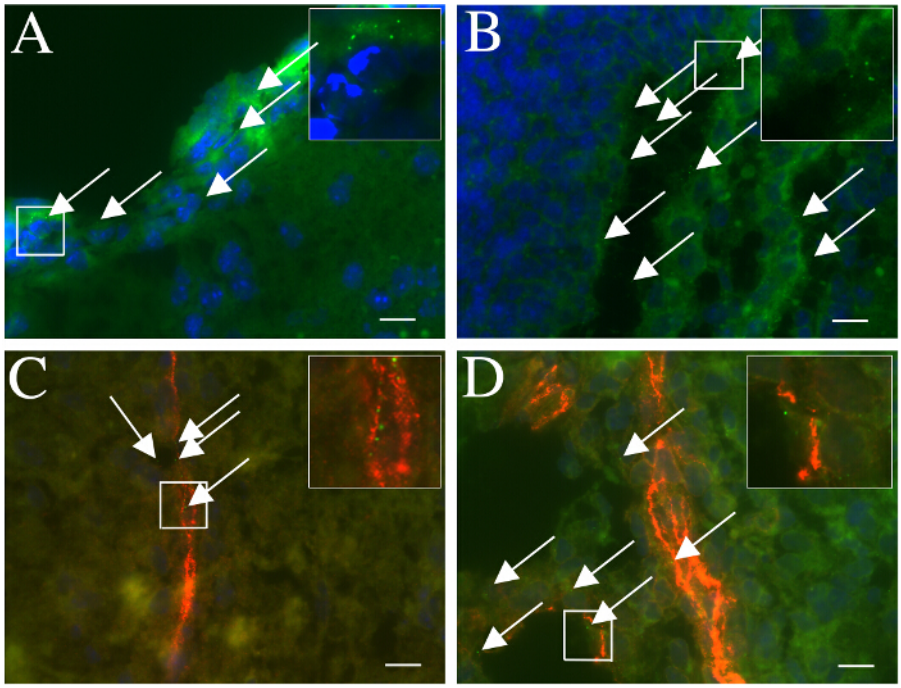

Figure 6: Observation of Virus Particles in the Acute Infection Phase. (A, B) EGFP dot signals (M32-EGFP-MCMV particles) are mainly observed in the MA (indicated by arrows; **A)**, choroid plexus, and SVZ (indicated by arrows; **B)** at 2 hr post ICV injection. The white squares are magnified as the enlarged inserts in the top right of **(A)** and **(B)**. Scale bar: 20 µm. **(C, D)** At 3 hr post IV injection, the MCMV particles (green, indicated by arrows) are found inside and outside of the vascular area of the parenchyma **(C)**, choroid plexus, and SVZ **(D)** (red: CD31-positive cells). The white squares are magnified as the enlarged inserts in the top right of **(C)** and **(D)**. Scale bar: 20 µm. Please click here to view a larger version of this figure.

Discussion

In animal models, ICV, intraperitoneal, direct placental, and IV infections have been used to study the pathogenesis of viral encephalitis. We focused on the ICV and IV injection models of neonatal mice for the simplicity of the procedures and the benefit of direct injection of particles into the target region. Although intraperitoneal infection is an easy method, viral particles spread systemically via an indirect process[5,24]. Direct placental infection is a good method to study embryonic systemic infection. However, this method requires special training to yield stable results and has a low success rate. The infection method is also an indirect process through the placenta and it is difficult to control the quantity of injection into the fetus at each trial[6]. As the neonatal mouse is very susceptible to viruses such as CMV, in this study we could compare the

distribution of viral particles and that of infected cells at the very early phase of infection. The ICV injection model is beneficial to study the infection behavior that bypasses the BBB[25], whereas the IV infection model is a systemic infection model resembling natural infection including the interaction of the virus with the BBB.

With the ICV injection method, we made sure to penetrate at least 2 mm deep into the skull. The needle was kept inside the cerebral ventricles for about 10 - 20 sec to prevent backflow from the ventricles[26]. When done carefully, ICV injection is a fast and easy *in vivo* method. However, sometimes inaccurate injections including unwanted backflow or deep/shallow penetration of the needle to the brain may happen. Careful procedures and observations post-injection are required to perform this protocol successfully. Considering the injection errors, we only focused on the distribution patterns of the particles and not on the quantity, and did not observe the treatment effects of drugs or functional antibodies in our study.

There are several procedures to perform the murine neonatal IV injection, through several sites such as superficial temporal veins, external jugular vein, and retro-orbital sinus. The superficial temporal vein of neonate is easily visualized by using a transilluminator and magnification lens. Injection can be completed by a single experienced individual with a high success rate.We chose the superficial temporal vein as the site of IV injection. The IV injection method requires some training such as positioning the neonatal mouse and the injection procedure. The head of the neonatal mouse should be positioned with tape for the temporal vein to be on top and flat. The 35 G needle should be inserted into the vessel far enough that the tip of the needle is completely surrounded by the vessel. A transilluminator and magnifying glasses are helpful tools to clearly visualize small veins. Injection of large volumes up to 100 µl should be infused slowly to confirm the flow of the contents. In case of failure of the injection at first trial, the other side of the temporal vein is available for another trial. After injection, pups experience minimal distress and recover quickly. It is not clear why an average of one out of 20 pups experience major distress and do not recover. However, the low rate of occurrence of this event minimally damages the whole experimental design.

As the IV injection method has a high success rate (more than 80%), we could compare the distribution pattern with and without systemic treatments such as drugs or functional antibodies. We have previously conducted an experiment where neonatal mice were injected with anti-β1 integrin functional blocking antibodies (50 µl at 20 µg/g) or isotype control antibodies (50 µl at 20 µg/g) into the superficial temporal vein of neonatal mice. One hour post antibody injection, 5×10^6 PFU (50 µl) of MCMV were infused into the other side of the temporal vein. The number of MCMV-positive cells per section of hamster anti-rat CD29 (β1 integrin chain, clone Ha2/5)-treated brain were significantly lower when comparing with that of isotype antibody-treated brain[8]. Using the same method in future investigations, the effects of drugs or neutralizing antibodies against viruses may be determined by observing the distribution of viral particles and infected cells.

Disclosures

The authors have nothing to disclose.

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